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(Direktor: Prof. Dr. J. A. Auer)

Establishment of a Method to Isolate and Culture Grey-Horse Melanoma Cells

Inaugural-Dissertation
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vorgelegt von
Nadine Metzger
Tierärztin
von Kirchberg (SG)

genehmigt auf Antrag von
Prof. Dr. J. A. Auer, Referent
Prof. Dr. Dr. M. O. Hottiger, Koreferent

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»Ring the bells that still can ring
Forget your perfect offering
There is a crack in everything
That's how the light gets in.«
–Leonard Cohen

Thank you for being there and believing in me:

Andrea O., Andrea M., Annika, Aurora, Christina, Claudia, Colin, Dee, Emma, Eva, Goya, Harriet, Jan, Joanne, Judith, Julie, Ken, Luzi, Lydia, Nicole, Notker, Marianne, Monika, Rainer, Renata, Renato, Roman, Ronny, Sabrina, Sara, Silvie, Stephanie, Tanja, Tina, Toni, Yuka.

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1. Summary

Routine isolation of grey horse melanoma cells and establishment of cell lines is the basis for any successful investigation of an effective modern therapy of this very common tumor in horses. Cell culture is the method to grow cells in a synthetic environment. Melanoma cell cultures exist of melanomas in swine, dogs, cats, mice, fish, and humans. No equine melanoma cell cultures have been described to date. In this report, a method to cultivate grey horse melanoma cells is described. Seven of 17 melanomas from 14 horses (success rate: 41%) were successfully transformed into a permanently growing cell line by enzymatic digestion with dispase and collagenase. The original tumors of successful cell lines were excised from the perianal region, tail base, and head. The melanomas were confirmed histologically and examined for association with successful/unsuccessful outcomes of the cell culture. Adherence was observed after 2-18 days. Confluency was reached after 10-100 days. The cells grew as adherent monolayers. They were of spindle shape and had long dendritic processes. Pigment production was evident. It accumulated extracellularly in the center of cell clusters. Successful isolation of grey horse melanoma cells is possible with this method. Further experiments to characterize the cells and to improve the isolation protocol are recommended.

2. Introduction

Equine melanoma is a common dermal tumor of grey horses.[1, 2] The term *equine melanocytic* tumor is synonymous with *equine melanoma*. The prevalence of this tumor is up to 15% of equine skin tumors.[3] It is estimated that 80% of grey horses over 15 years of age develop melanoma.[4] The high incidence of melanoma in grey horses is thought to be related to changes in melanin metabolism associated with aging.[5-8] Usually they appear at the age of 5 years, corresponding to the coat color changes. There is a loss of melanin pigment in the hair follicles, which results in a grey hair appearance.[3, 4, 9] No gender predilection exists.[3] Primary tumors are most frequently found in the perineal region, ventral tail, and external genitalia. Less common sites are limbs, neck, region of the parotid salivary gland, head, and eyes.[3, 10-12] They tend to grow slowly and usually are multicentric and locally invasive. The size varies from less than 1 cm to 20 cm in diameter. Metastases can occur to virtually any site with regional lymph nodes and the lungs as the most common internal organ systems affected.[11, 13]

There are 4 syndromes of equine melanoma. Three of them have a potential for metastasis.[10] The first two categories are dermal melanoma and dermal melanomatosis, which are histologically similar, but clinically different. (i) *Dermal melanomas* are solitary, discrete lesions in which surgical excision is usually curative with a low incidence of metastasis.[1] (ii) *Dermal melanomatosis* is the term used for multiple, coalescing, not surgically excisable lesions occurring in typical locations in grey horses older than 15 years. They are reported to have a high incidence of metastasis.[10] Both types of melanomas are difficult to distinguish from each other and their metastatic potential can not be determined by histologic analysis.[11] (iii) *Anaplastic malignant melanomas* occur in non-grey horses and commonly develop lethal organ metastasis.[14, 15] (iv) *Melanocytic nevi* are benign, discrete, superficial, pigmented lesions in younger horses of all colors. Surgical excision is usually curative.[10] Approximately 95% of melanocytic tumors are benign at the time they are identified in a horse.[16] However, two-thirds are thought to progress to malignancy and are capable of widespread metastasis.[17, 18] It has been reported that melanomas in non-grey horses are more likely to be malignant.[19-21] Diagnosis of equine melanoma is based on clinical, cytologic, and histopathologic examination.[16] Frequently melanomas are not treated because of the initial benign behavior, slow-growing nature of the neoplasm, size, proximity to major vessels and vital structures, or extensive local invasion.[11] There may be an increased risk of metastasis after mechanical stimulation of a melanoma.[22] Treatment options include surgical excision,[23-25] partial tail

amputation,[11] cryotherapy,[26] oral systemic therapy with cimetidine,[11, 27, 28] intralesional chemotherapy with cisplatin,[29] and treatment with unspecific immunomodulators such as BCG (Bacillus Calmette-Guérin).[5] Except for total surgical excision of small and well demarcated melanomas,[30] which have a good prognosis, treatment success is rather poor or palliative at the most. In recent years human and veterinary tumor treatment research has focused on autogenous vaccines. A crude whole-cell melanoma vaccine has been used with mixed results in managing equine neoplasia.[5, 11] Intratumoral injection of interleukin-18-DNA and partially interleukin-12-DNA had an inhibitory effect on melanoma growth in grey horses.[31, 32] Prognosis for complete remission usually is grave. Problems arise when primary tumors or metastases compress internal organs and lead to problems with physiological function such as respiration, defecation, or nerve conduction.[11, 33-40] Pulmonary or visceral metastases can be life-threatening.[3] Large superficial tumors may ulcerate and become infected. Interference of external tumors with riding gear may render a horse useless for its intended use.

To investigate immunobiologic and pathobiologic features of equine melanocytic tumors it is necessary to dispose of equine melanoma cell lines. Readily available equine melanoma cell lines would provide good resources for further investigations on equine melanoma therapy. Cell culture is a method to grow cells of organic origin in a synthetic environment.[41] A *primary* cell culture consists of cells that derive from an original, intact tissue. Primary cell cultures typically will have a finite life span in culture. After the primary cell culture is sub-cultured it is termed a *cell line*. A cell line can become a permanently established cell culture that will proliferate indefinitely with the appropriate maintenance. One method to isolate cells from the original tissue is enzyme digestion with proteolytic enzymes. Culture conditions such as growth medium, temperature, oxygen concentration, carbon dioxide concentration, pH, and osmolality vary for each cell type. In general, the closer the artificial conditions mimic the original environment the better the cells grow. Some cells grow without attachment to a surface and proliferate in suspension. However, most cells are anchorage dependent, meaning they require attachment to a substrate to proliferate. The culture medium is probably the most important factor in culturing animal cells. It provides nutrients, pH, and osmolality. A large number of various cell culture basal media are available commercially. It is common practice to supplement the basal medium with additives such as animal sera, amino acids, and antibiotics.[42] Optimal culture conditions for cell growth of mammalian cells are a pH of 7.2-7.5, an osmolality of 280-320 mOsm/kg, a temperature of 35-37 °C, and a carbon dioxide

concentration of 2-5% in air.[43] The use of a humidified incubator with 5% CO_2 usually provides suitable environmental conditions.

Establishment of permanent melanoma cell lines has been reported in various animal species. A total of 66 cell lines derived from 21 porcine melanotic lesions were studied over their life spans of up to 14 months.[44] The lesions were of cutaneous and visceral origin. Eight canine melanoma cell lines were established from tissues of 6 dogs with oral melanomas and metastatic lesions to liver and lymph nodes.[45] They had been maintained in culture for longer than 1 year. Seven other attempts to establish cell lines were unsuccessful because either they did not grow or fibroblast overgrowth was present. Other canine melanoma cell lines were established from canine skin melanomas,[46] oral melanomas,[47] and lung metastases.[48] Of the infrequent cat melanoma 3 cell lines were established from the primary throat tumor and from metastatic lung and heart tumors of the same animal.[49] The author mentioned that long-term cultures of feline tumors are difficult to establish because of cytopathic viruses in cat tissue that destroy the cultures. Numerous murine cell lines are available commercially.[50] An amelanotic melanoma obtained from a tail fin of a platyfish-swordtail hybrid fish was successfully transformed to a cell line, which was maintained for over 2 years.[51] Human uveal melanoma cell lines were established and maintained in culture for up to 2.5 years by several investigators.[52-56] Reports of cell lines from human primary melanomas and metastases are abundant and many of these cell lines are available commercially.[50, 57-70] To the authors' knowledge, there is no information in the current literature on routine *in vitro* growth of equine melanoma cells. Doyle et al. mention one cell line with over 20 passages established from a tumor that was histologically diagnosed as a melanoma when they studied the cytotoxic effects of various drugs on equine cells *in vitro*.[71] Apparently the tumor was taken from a Thoroughbred yearling filly. However, it is unclear if the horse was of grey or solid color and there is no information on the isolation method or characterization of this cell line.

The objectives of this study were (a) to develop and establish a method for routine isolation and cultivation of grey horse melanoma cells, and (b) to characterize cellular growth properties *in vitro* and describe the morphology of seven permanently growing grey horse melanoma cell lines. This study was a preliminary study for the long-term goal of developing an autologous tumor vaccine for equine melanoma. Once a reliable protocol for equine melanoma cell cultures will be established, studies on transfection of melanoma cells with gene constructs

and subsequent reinjection into the patient the cells originally were derived from may be pursued.

3. Materials and Methods

a) Solutions

Solutions were prepared according to the following instructions:

Roswell Park Memorial Institute 1640 (RPMI 1640) Rinse Solution

RPMI 1640 500 ml,[a] L-Glutamine 10 ml (=4 mM/ml maintenance medium),[b] Pyruvate 5 ml (=0.01 mM/ml maintenance medium),[c] Antibiotic Mixture 5 ml (=Penicillin G 50 units/ml, and Streptomycin sulphate 50 units/ml maintenance medium, respectively)[d]

Roswell Park Memorial Institute 1640 (RPMI 1640) Culture Medium

RPMI 1640 Rinse Solution 500 ml, heat inactivated FCS 50 ml (=10%)[e]

Dispase Solution

PBS 63 ml,[f] 0.1 g Dispase (yields 2 units/ml dispase solution)[g]

Tris-buffered saline (TBS)

NaCl 8 g,[h] Tris-Base 3 g,[i] KCl 0.2 g,[j] dH_20 1 l, adjust to pH 7.4 with HCl[k]

Phosphate-buffered saline (PBS)

NaCl 8 g, KCl, 0.2 g, Na_2HPO_4 1.44 g,[l] KH_2PO_4 0.24 g,[m] dH_20 1 l, adjust to pH 7.4 with HCl

Calcium Stock Solution

$CaCl_2$ 554.93 g,[n] TBS 100 ml[o]

Collagenase Stock Solution

TBS 3.04 ml, Collagenase 0.1 g (yields 4112 units/ml Collagenase Stock Solution)[p]

Fetal Calf Serum (FCS)

Heat inactivation in a water bath at 56°C for 30 minutes

Stop Solution

NaCl 1.35 g, Tris Base 0.6 g, EDTA Salt 0.03 g,[q] dH_2O 0.1 l, adjust to pH 7.4 with concentrated HCl

Antibiotic Mixture

Penicillin G (5000 units/ml), Streptomycin sulphate (5000 units/ml) in normal saline

Work in the cell culture laboratory was carried out under a laminar flow hood. All instruments and materials used in the disaggregation process were sterile. Surgical instruments were autoclaved before use and solutions were filtered through filter units[r] or filter discs.[s] Before use all media and solutions were warmed in a water bath at 37 °C.

b) Horses and collection of samples

14 healthy grey horses were eligible to elective standing excision of one melanoma (Table 1). Three horses had removed a second melanoma from a different location at a second visit to the clinic. The horses were placed in stocks and sedated with detomidine[t] (0.01 mg/kg IV) and butorphanol[u] (0.02 mg/kg IV), followed by a continuous rate infusion of detomidine adjusted as needed. Immediately before surgery they were administered flunixin meglumine[v] (1 mg/kg IV). Tumors with a size of 4 to 20 mm in diameter, good surgical accessibility, and small risk of wound healing complications were selected. The hair was clipped and the surgical site prepared for aseptic surgery. Local anesthesia was provided by peritumoral infiltration of lidocaine 2%[w] SC. The melanoma was excised applying to standard surgical technique. The volume of the excised tumor material was estimated. Immediately following excision a peripheral slice of tumor tissue was placed in 4% formalin[x] and submitted for histologic examination. The remaining tissue was placed in sterile Dulbecco's phosphate buffered saline (D-PBS)[y] containing 1% Antibiotic Mixture at room temperature. It was immediately transported to the cell culture laboratory. Time from excision to further processing was 15 to 20 minutes in all excised tumors. An equine melanoma cell line (kindly provided by Dr. M. Seltenhammer, University of Veterinary Medicine, Vienna, Austria) was used as a control and was grown under the same conditions as the isolated tumor cells.

Table 1: Signalment of horses, location of tumor, and result of experiment.

Melanoma No.	Horse No.	Age [yrs]	Sex	Breed	Site	Result
M1	a	16	mc	Irish WB	Head	G
M2	b	17	mc	Swiss WB	Pectoral	NG
M3	a[#]	16	mc	Irish WB	Head	C
M4	c	20	mc	Anglo Arabian	Perianal	G
M5	d	21	mc	Irish WB	Perianal	C
M6	e	13	mc	Swiss WB	Perianal	C
M7	f	26	mc	Andalusian	Perianal	C
M8	g	10	mc	Lusitano	Perianal	G
M9	h	14	f	Arabian	Perianal	G
M10	i	12	mc	Swiss WB	Perianal	NG
M11	j	9	mc	Dutch WB	Head	NG
M12	i[#]	12	mc	Swiss WB	Perianal	G/NG*
M13	f[#]	26	mc	Andalusian	Perianal	NG
M14	k	12	mc	Andalusian	Perianal	G
M15	l	8	f	Swiss WB	Dorsal tail	NG
M16	m	6	m	Andalusian	Perianal	G
M17	n	9	f	Arabian	Ventral tail	G

Abbreviations: m=male intact, mc=male castrated, f=female, WB=warm blood, G=cell growth, NG=no cell growth, C=contamination, [#]second visit, *initial growth followed by cell death for unknown reason.

c) Method of cultivation

Working under absolutely aseptic conditions is very important when dealing with primary cell cultures. Any handling of the tumor or cell culture should only be carried out in a laminar flow hood. All instruments used must be sterile. Wearing gloves and wiping anything carried into the laminar flow hood with ethanol is recommended. Melanoma cells were dissociated from the tumors by two slightly different enzymatic digestion protocols. Both protocols were employed simultaneously on each tumor. Protocol A included incubation with the enzyme dispase for 3 hours, followed by incubation with the second enzyme collagenase for 2 hours. Protocol B was identical except for longer incubation time: Incubation with dispase and collagenase were continued for one additional hour, respectively. The melanoma was placed in a tissue culture dish[z] and rinsed with D-PBS containing 1% Antibiotic Mixture. The mass was

trimmed free of overlying epidermis, surrounding connective tissue, and fat tissue. This was done for two reasons: To get rid of any potentially infected tissue (epidermis) and to obtain pure melanoma tissue with only a minimal amount of other cell types. Each tumor was split in two pieces. With scalpels and scissors the cleaned halves were minced into very small pieces. The larger the total surface the more cells can be isolated by enzymatic digestion from the solid tissue. When processing the tissue, care must be taken to work quickly and to avoid any unnecessary delay until the cells are placed in the culture medium. Pieces were transferred in equal parts to two 50 ml Falcon tubes.[aa] Each falcon tube had previously been filled with 5 ml Dispase Solution (=10 units), 5 ml RPMI 1640 Rinse Solution, and 0.1 ml Antibiotic Mixture each. The lids of the Falcon tubes were placed slightly loosened to allow for gas exchange. They were placed in a water bath at 37 °C for 3 and 4 hours, respectively. Every 30 minutes they were agitated gently by hand. After its respective incubation time each tube was removed from the water bath and the lids were closed securely. This was followed by centrifugation at 2000 rpm for 5 minutes at room temperature. The supernatant was discarded by suction. The remaining pellet was suspended in 13.35 ml Tris-buffered saline (TBS), 1.5 ml Calcium Stock Solution, 0.15 ml Collagenase Solution (= 617 collagen digestion units), and 0.15 ml Antibiotic Mixture. After thorough mixing of the contents the lids of the Falcon tubes were slightly loosened again. The tubes were replaced in the water bath for 2 and 3 hours, respectively. They were centrifuged and the supernatant was removed. The pellet was suspended in 2 ml Stop Solution at 4 °C, left aside for 5 minutes at room temperature, centrifuged at 2000 rpm for 5 minutes, and the supernatant was removed. The pellet was suspended with 10 ml RPMI 1640 Culture Media, centrifuged at 2000 rpm for 5 minutes, and the supernatant removed. The last cycle was repeated twice. Before the last centrifugation the suspension was filtered through a 70 µm pore filter.[bb] The final cell pellet was suspended with 2 to 4 ml RPMI 1640 Culture Medium containing 10% FCS, 2% glutamine, 1% pyruvate, and 1% Antibiotic Mixture. Depending on the cell density the suspension was added to 2 to 4 tissue culture dishes containing 10 ml RPMI 1640 Culture Medium. The tissue culture dishes were transferred to a humidified incubator gassed with 5% CO_2 at 37 °C.

All cultures were examined daily for evidence of growth and adherence, using an inverted light microscope. RPMI 1640 Culture Medium was replaced 1-3 times a week. When cultures obtained >90% confluency, cells were sub cultured diluted at ratios of 1:2 or 1:3. All culture media was discarded by suction. The adherent cells were washed with 10 ml D-PBS. All fluid

was removed by suction. One ml of 0.5% trypsin-0.2% Na_2Ethylenediamine tetra-acetic acid (EDTA) in Hanks' Balanced Salt Solution (HBSS) without Ca^{2+}, Mg^{2+}, and phenol red was added to the dish.[cc] It was placed in the humidified incubator at 37 °C and repeatedly observed under the inverted light microscope until complete detachment of the cells was detected. The enzymatic reaction was stopped by adding 5 ml of RPMI 1640 Culture Medium to the tissue culture dish. The cell suspension was transferred to a 15 ml Falcon tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed by suction. The pellet was suspended in cell culture medium and added to 2 to 4 new tissue culture dishes as described above.

Samples of all generations of each cell line were frozen in liquid nitrogen. The protocol for this procedure followed the sub culturing-protocol described above. Instead of adding cell culture medium to the cells, they were suspended in Cell Culture Freezing Medium-DMSO.[dd] The suspension was dispensed into multiple cryotubes[ee] depending on the cell density. The cryotubes were placed on ice for 30 minutes, then transferred to a -70 °C freezer for 24 hours, and finally stored at -196 °C in liquid nitrogen until further use. For renewed activation of cell growth or further experiments the liquid nitrogen stabilates were removed from the liquid nitrogen freezer and immediately thawed in a warm water bath at 37 °C. The suspensions were seeded on a tissue culture dish with RPMI 1640 Culture Medium at 37 °C as described above.

d) Characterization of tumors and cell lines

Localization: To document external morphology, tumors M3-M11 and M15-M17 were photographed *in vivo* before excision.

Histology: Of tumors M2-M17 a peripheral tissue slice was placed in 4% formalin immediately following surgical excision. M1 was part of a preliminary cell culture study when no histology examination was initiated yet. Four-µm-thick, paraffin embedded tissue sections were obtained and stained with hematoxylin and eosin for histopathology. They were examined for the following characteristics: Ulceration of the epidermis, encapsulation of the tumor, shape, symmetry, adnexal involvement, junctional activity, pattern of growth (invasive, expansive), vascularization, presence of necrotic areas, cellular composition, presence of inflammatory cells and melanophages, apoptosis, mitotic figures, pigmentation, size and shape of the cells, size and shape of the nuclei, and presence of nucleoli. Tumor classification was not performed.

Light microscopy: Cells were photographed through a phase-contrast microscope by using an inverted microscope equipped with phase-contrast lenses as they grew *in vitro*. Cell morphology, cell size and shape, alignment of cells, group pattern, interactions between cells, pigment production, and any other interesting feature were documented.

4. Results

a) Success rate of establishing melanoma cell lines in culture

A total of 17 tumors were harvested and processed. Establishment of a cell line was successful in 7 cases (M1, M4, M8, M9, M14, M16, M17) from 7 horses (Table 2). One tumor (M12) grew in very few and large individual cells, which died for unknown reasons after 2 divisions. Five tumors completely failed to grow (M2, M10, M11, M13, M15). Four cell cultures had to be discarded because of contamination in the first week after isolation and before any cell growth was observed (M3, M5, M6, M7). Bacteriologic examination of culture media revealed contamination with *Streptococcus spp*. The success rate to establish continuous cell lines from primary tumors was 41% when counting all cultures (7/17), and 54% when counting the non-contaminated cultures only (7/13). Since no statement can be made about success or failure of the cell cultures that had to be discarded because of contamination those cultures will not be further discussed in the sections below.

b) Morphology of tumors

The morphology data of the successful cell lines are summarized in Table 2. The tumors originated from the perianal region (5/7), the head (1/7), and the ventral tail base (1/7) (Figs. 1-3). The tumors were located subcutaneously (4/7), intradermally (1/7), or both (2/7). The volume of the tumor tissue available for disaggregation corresponded to about two thirds of the original tumor material excised. It ranged from 0.3-5.0 cm^3. Most of the tumors were perfectly round in shape (5/7), some were rather oval (1/7) or irregularly shaped (1/7). All tumors, successful and unsuccessful alike, had a deep black surface on cut sections. Five tumors possessed a rubber-like consistency and were hard to cut. Two tumors were firm on palpation but rather soft to cut.

Table 2: Description of tumors that yielded successful cell lines.

No.	Site	Layer	Volume [cm³]	Shape	Color	Solidity	Demarcation
M1	Head	sc	5.0	oval	black	very firm	good, encapsulated in connective tissue
M4	Perianal	id	0.5	round	black	very firm	diffuse, mixed with large amount of connective tissue
M8	Perianal	id, sc	1.0	round	black	soft	good, easy to cut
M9	Perianal	sc	0.5	round	black	soft	good, easy to cut
M14	Perianal	sc	1.0	round	black	firm	good
M16	Perianal	sc	1.0	round	black	firm	good
M17	Tail base	id, sc	0.3	irregular	black	firm	diffuse

Abbreviations: sc=subcutaneous, id=intradermal

Figures 1-3: Examples of melanomas *in vivo*. All three tumors were grown successfully.

(From left to right) Fig. 1: Melanoma 4. Multiple, severe perianal melanomas. Fig. 2: Melanoma 8. Multiple, nodular perianal melanomas. Fig. 3: Melanoma 17. Melanoma is located on the ventral tail base. Picture is taken immediately before excision. The skin is incised already.

Tumor M12, which grew initially and then died, was located in the perianal region. It was a small tumor with a volume of only about 4 mm^3. The tumor was round, firm, and hard to cut. Very little cell material was yielded after enzymatic digestion. Two of the 5 unsuccessful tumors originated from the perianal region, the remaining 3 tumors were from the head, dorsal tail, and pectoral region, respectively (Figs. 4-6). Two were located intradermally, one was subcutaneously, and one was both. An exception was tumor M2. It was very large (estimated 6x4x4 cm) and located in the pectoral region deeply embedded between muscle tissues at about 8 cm from the skin. Because of the size and location only a wedge biopsy of about 6 cm^3 was taken. The surface of the tumor was covered with connective tissue. It was very easy to cut, the central material of the tumor was almost liquid and there was very little intratumoral connective or fat tissue. Large amounts of free, heavily pigmented, necrotic material was present. M10 was round, had a volume of 1.5 cm^3, and revealed identical necrotic features as M2. M11 was round, 2 cm^3 in volume, firm with few connective and fat tissue. M13 was round, 1cm^3 large, with a lot of free pigmented material, and not well demarcated. M15 was 2 cm^3 large, round, firm, and hard to cut.

Figures 4-6: Examples of melanomas *in vivo* that could not be grown *in vitro*.

(From left to right) Fig. 4: Melanoma 10. Multiple, isolated perianal melanomas. Fig. 5: Melanoma 11. Single, isolated facial melanoma. Fig. 6: Melanoma 15. Single, lateral tail base melanoma. The surface is ulcerated.

c) Histologic examination

Six of the 7 successful cell lines originated from tumor material that was examined histologically (Figs. 7-12) On histologic examination all six melanomas were not encapsulated and appeared to grow invasively. One tumor had junctional activity (M9). Some masses consisted of more than one cell type. Cell morphology of the neoplastic cells varied from round, polygonal, to spindloid. Occasionally dendritic cells were identified. No apoptotic cells were present and no mitotic figures were observed in any of the melanoma cells. The cytoplasm contained little to abundant melanin. The nuclei were typically round to ovoid and euchromatic. They contained one or more prominent nucleoli. There was a mild degree of anisokaryosis and moderate polymorphism of the nuclei and cells. The histologic diagnoses were melanoma (M4), moderately malignant melanoma (M16, M17), and malignant melanoma (M8, M9, M14). All tumors were considered at risk for relapse and formation of metastasis.

All the unsuccessful tumors, including tumor M12, which died after two divisions, grew invasively (Figs. 13-18). M15 was the only tumor with ulceration of the epidermis. Three tumors were necrotic in the center (M2, M10, M13). None of the tumors were encapsulated. Four tumors contained large amounts of connective tissue. The tumor shape varied from oval to asymmetrical. Four tumors had adnexal involvement. Two tumors showed junctional activity (M11, M15). Vascularization was good in all tumors. No apoptosis was present. M15 had mitotic figures. Pigmentation was rather coarse except for M13, which was finely granulated. Cell shapes were similar to the successful tumors described above but none of the cells had a dendritic appearance. The cells were larger compared to the successful tumor cells. Nuclei, nucleoli, pigment content of the cells, and anisokaryosis were comparable to the successful melanomas. Histologic diagnoses were melanoma (M2) and malignant melanoma (M10-M13, M15). All tumors were considered at risk for relapse and formation of metastasis.

Figures 7-12: Histologic examination of successful tumors.

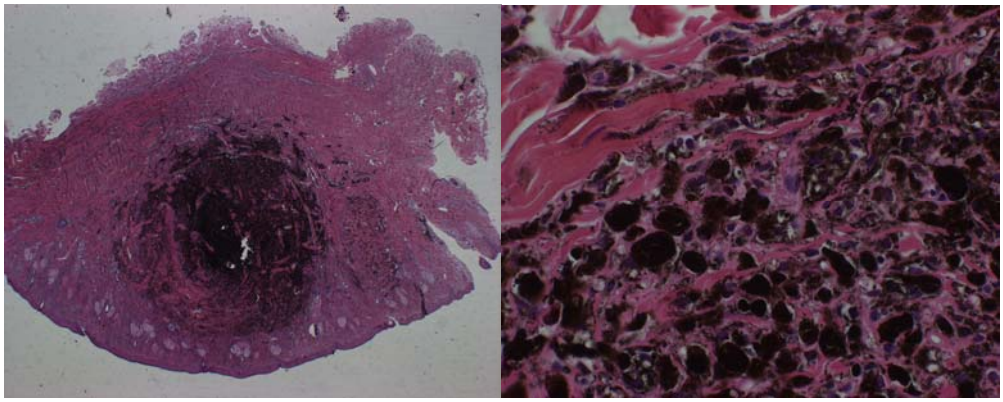

Fig. 7: Melanoma M4. 1x (left) and 40x (right)

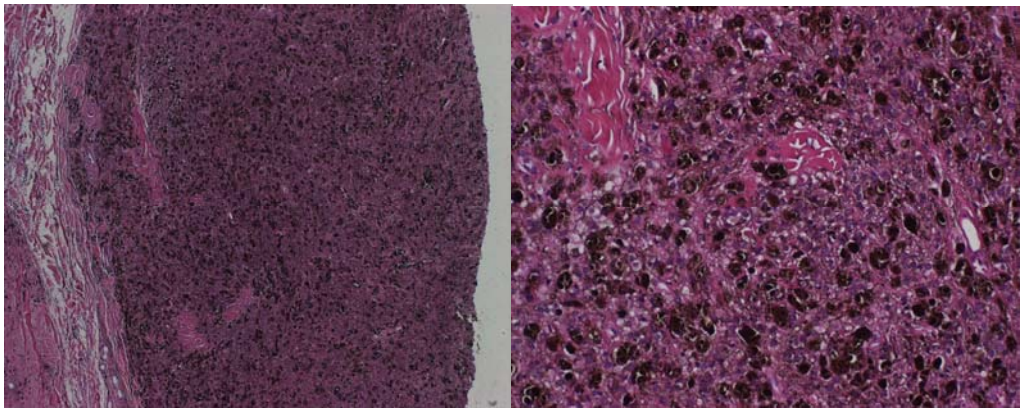

Fig. 8: Melanoma M8. 4x (left) and 20x (right)

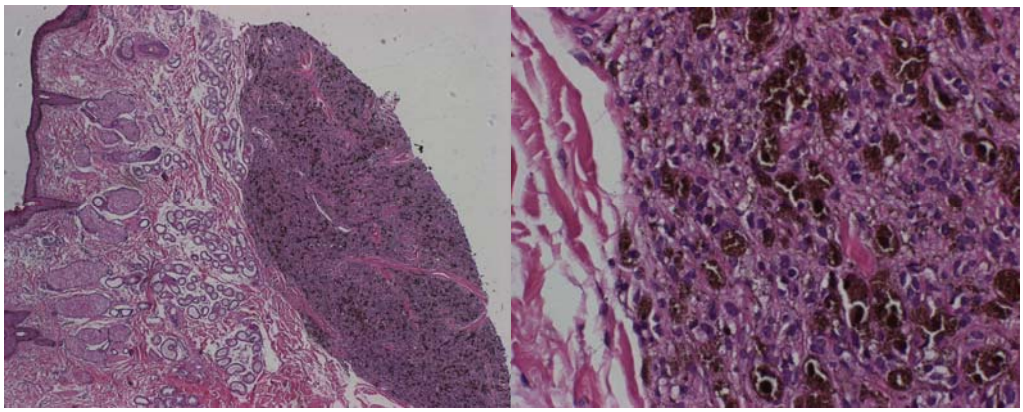

Fig. 9: Melanoma M9. 4x (left) and 40x (right). Junctional activity.

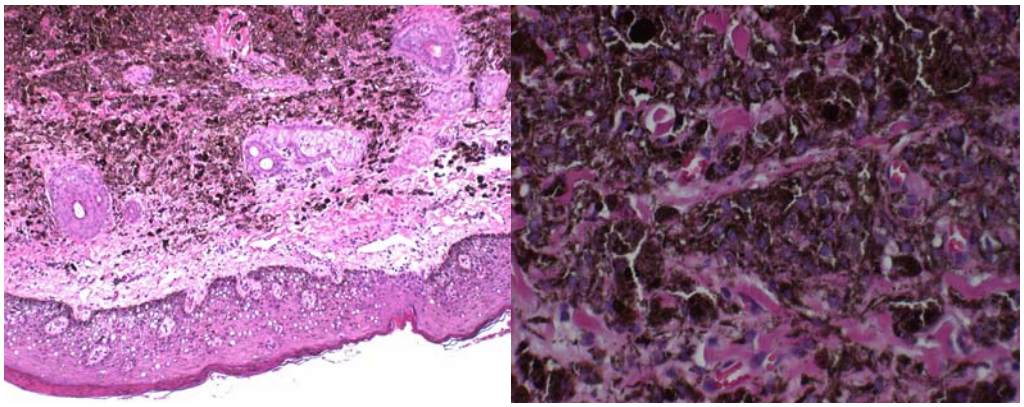

Fig. 10: Melanoma M14. 10x (left) and 40x (right)

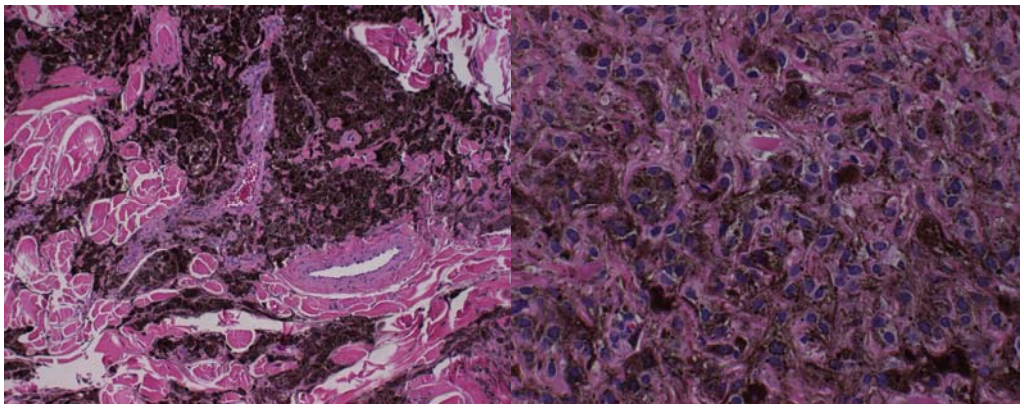

Fig. 11: Melanoma M16. 10x (left) and 40x (right)

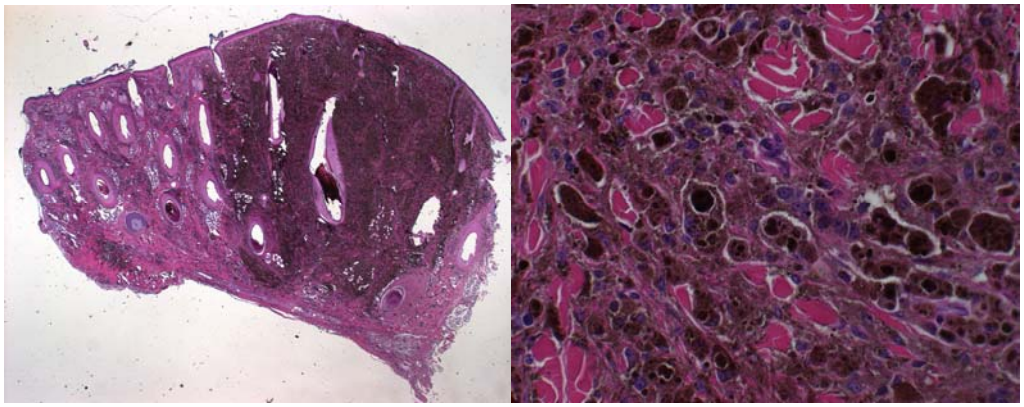

Fig. 12: Melanoma M17. 1x (left) and 40x (right)

Figures 13-18: Histologic examination of unsuccessful tumors.

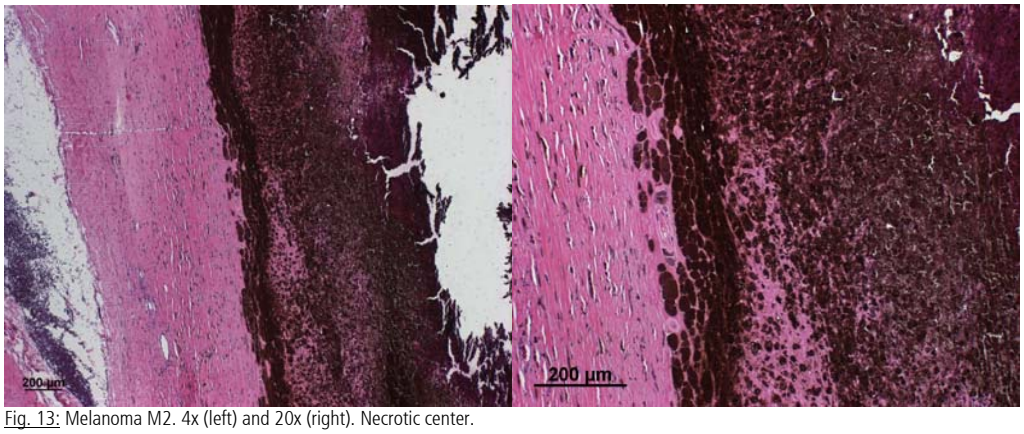

Fig. 13: Melanoma M2. 4x (left) and 20x (right). Necrotic center.

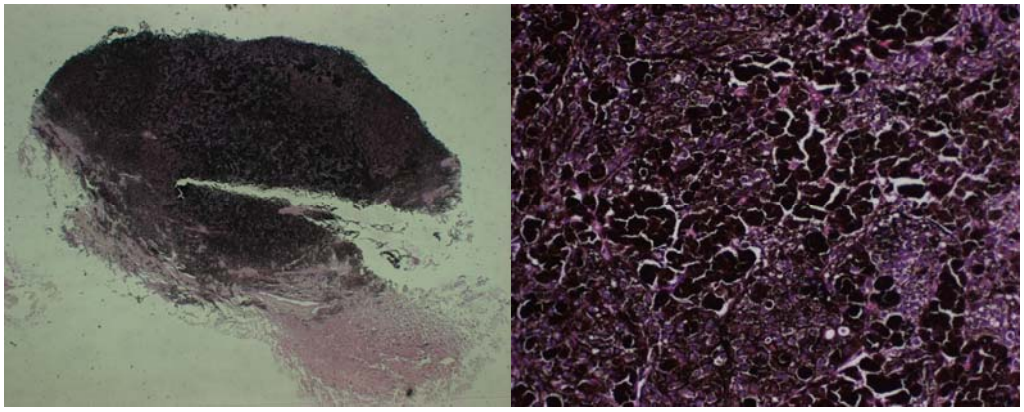

Fig. 14: Melanoma M10. 1x (left) and 20x (right). Necrotic center.

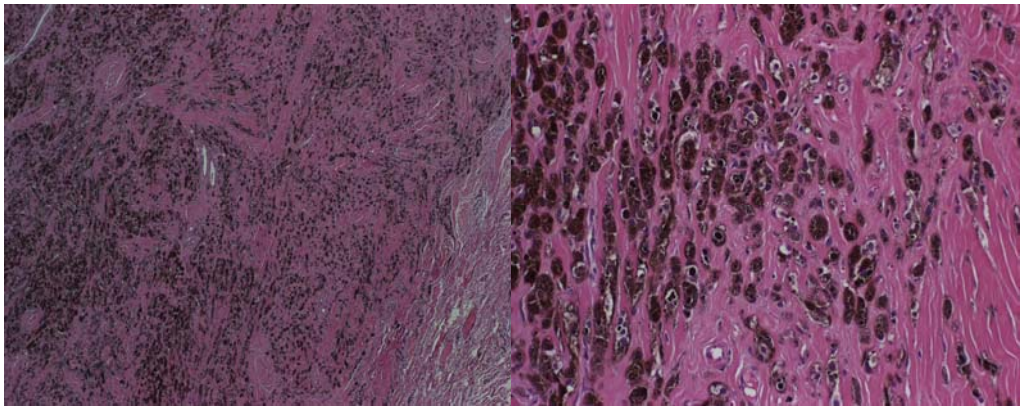

Fig. 15: Melanoma M11. 4x (left) and 20x (right)

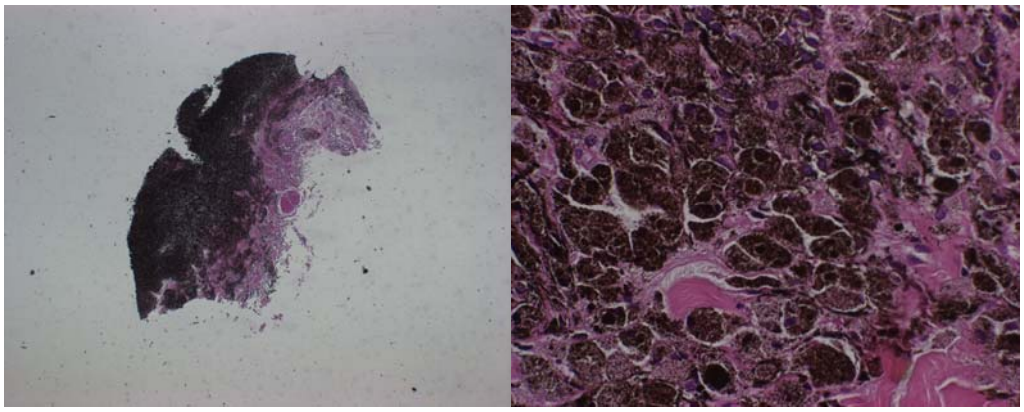

Fig. 16: Melanoma M12. 1x (left) and 40x (right)

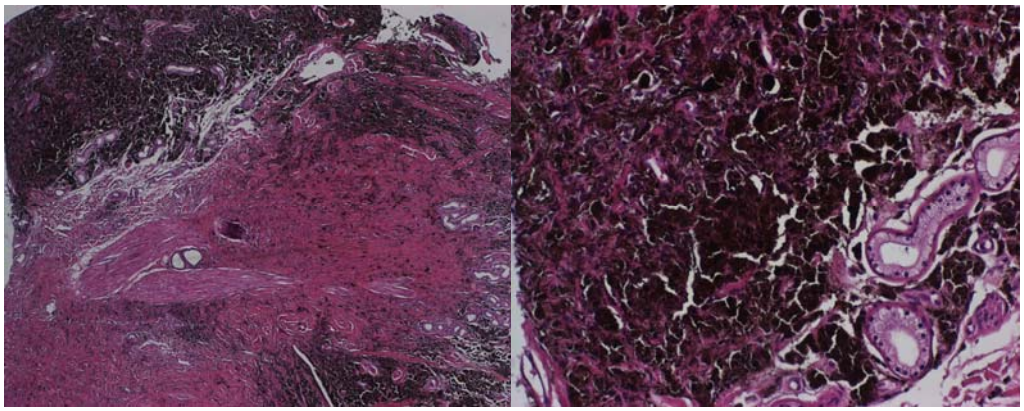

Fig. 17: Melanoma M13. 4x (left) and 20x (right). Necrotic center.

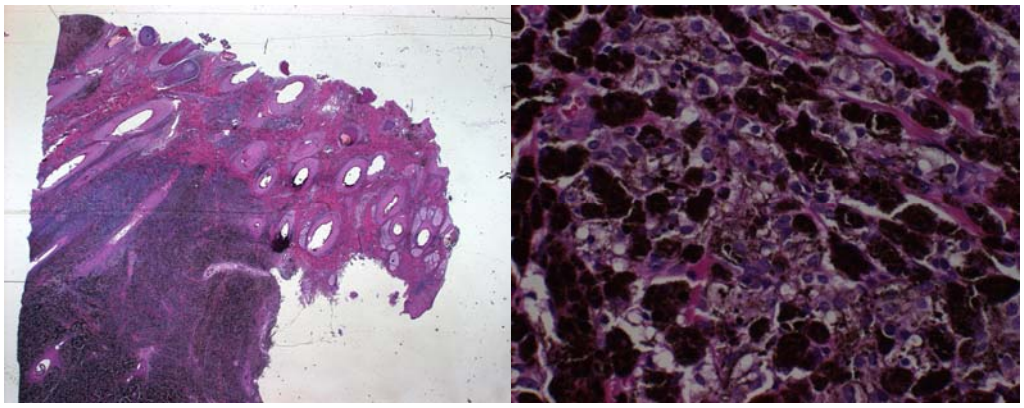

Fig. 18: Melanoma M15. 1x (left) and 40x (right). Ulcerated epidermis.

d) Cell culture growth and morphology

All successful cell lines grew as adherent monolayer cultures in tissue culture dishes. Cellular morphology when evaluated by an inverted microscope was similar in most cell lines. Cell lines M1, M4, M8, M14, M16, and M17 were comprised of spindle shaped to dendritic cells (Figs. 19-26). Some cells had very long dendritic processes. They had a low nuclear-to-cytoplasmatic ratio with round to oval nuclei, multiple dark nucleoli, and well-defined cytoplasmic borders. In cell line M9 an additional cell type to the ones described above was detected. Those cells were epithelioid and smaller than the dendritic cells. They grew in a denser pattern building sheet-like structures. Pigment production was evident in all primary cell cultures. The cells were of very faint light brown color. The cells grew in clusters with dark brown, granular accumulation of extracellular pigment in the center. Cell line M14 was unique in that a large percentage of its cells had intracytoplasmatic small melanin granules instead of the large extracellular deposits. It appeared that in general the cells became amelanotic and ceased producing melanin after being sub cultured. They were smaller and less dendritic compared to the primary culture. The control equine melanoma cell lines consisted of amelanotic, very small, polygonal cells that grew in sheets (Fig. 27).

Figures 19-27: Primary cell cultures *in vitro*.

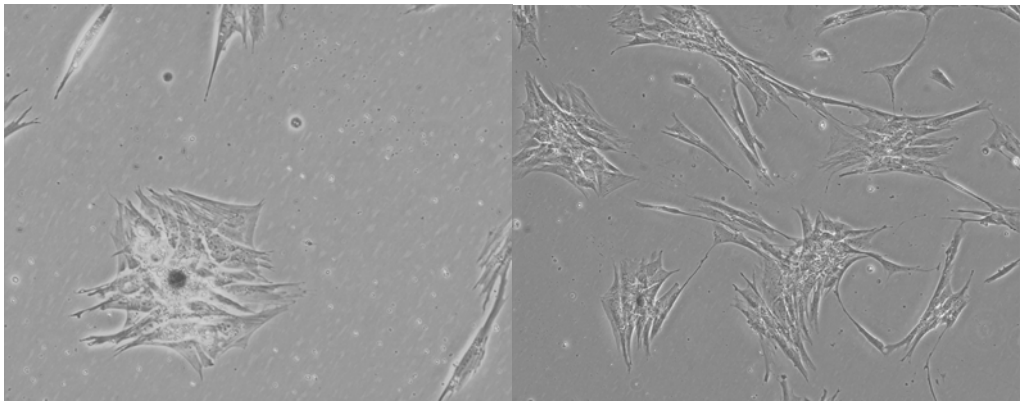

Fig. 19: Melanoma M1. Day 31. Cell clusters with central pigment deposits.

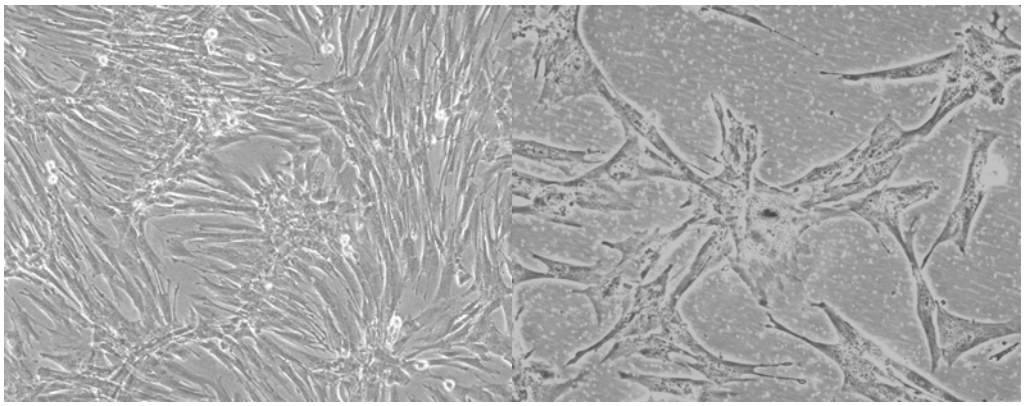

Fig. 20: Melanoma M4. Day 21. Cell clusters with central pigment deposits. Note long dendrites of cells.

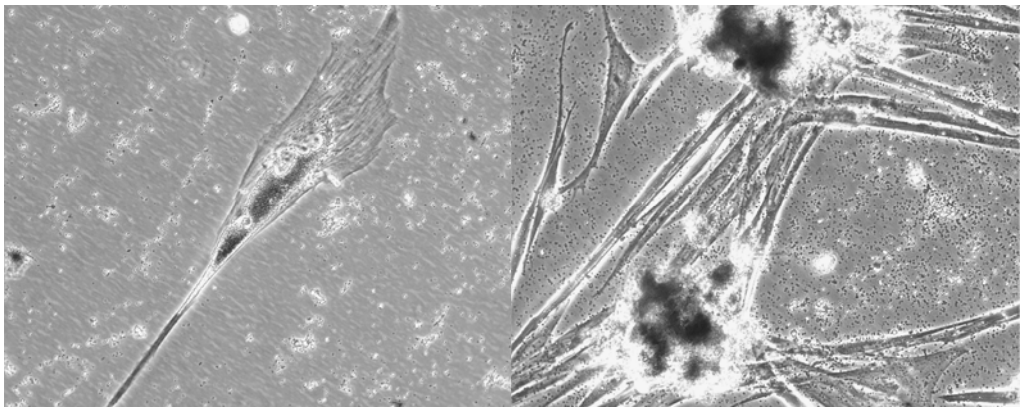

Fig. 21: Melanoma M8. Day 102. Large amount of dark pigment deposit (left). Single dendritic cell with intracellular pigment granules (right).

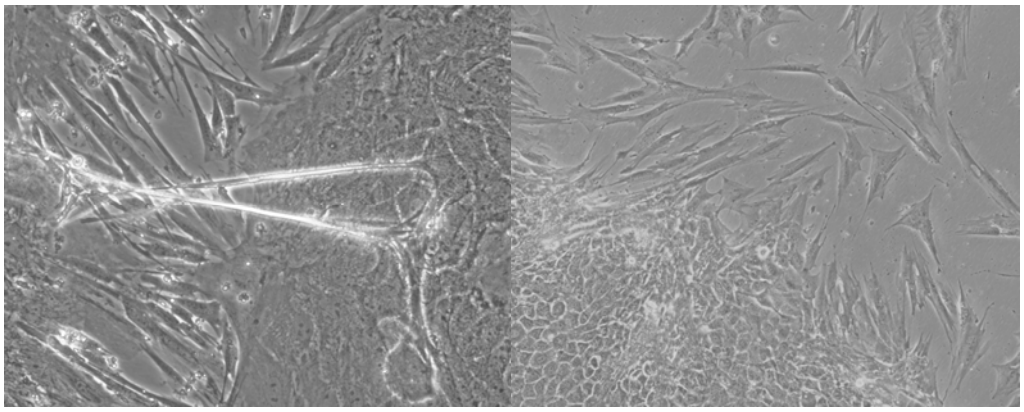

Fig. 22: Melanoma M9. Day 14. Long dendrite reaching out on top of other cells to make contact (left). Two different cell types: Epitheloid, round cells and spindloid, dendritic cells.

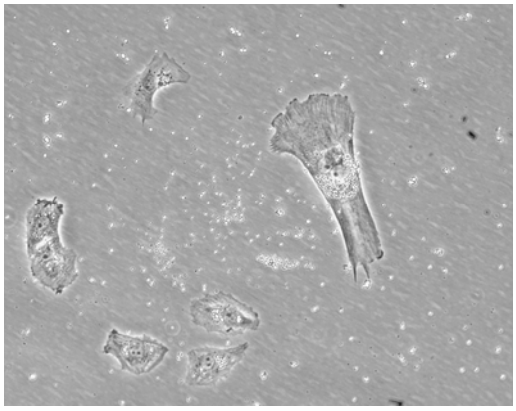

Fig. 23: Melanoma M12. Day 99. Large individual cells without dendrites and no clustering behaviour. Cells died after 2 divisions.

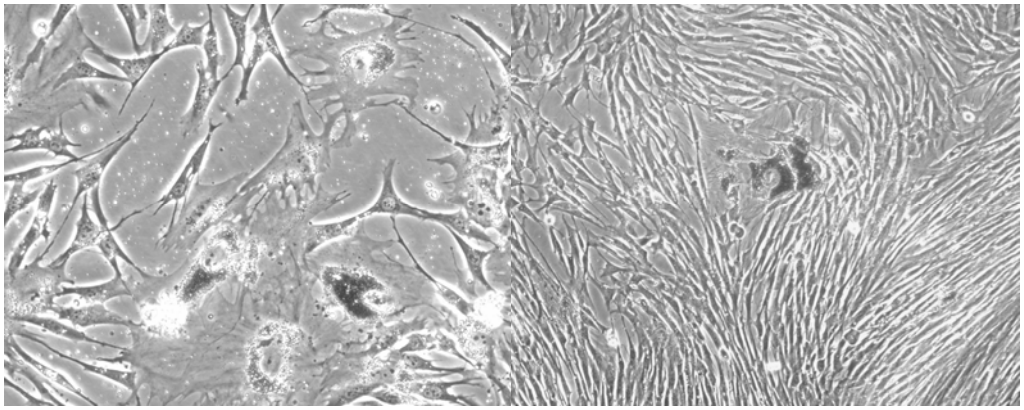

Fig. 24: Melanoma M14. Day 32. Large cells with intracytoplasmatic pigment granules. Other cells are smaller but also dendritic.

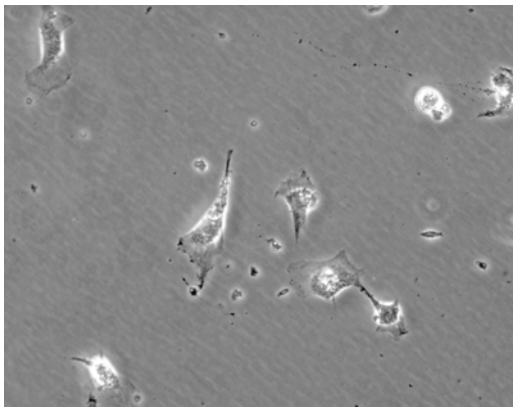

Fig. 25: Melanoma M16. Day 93. Small single cells.

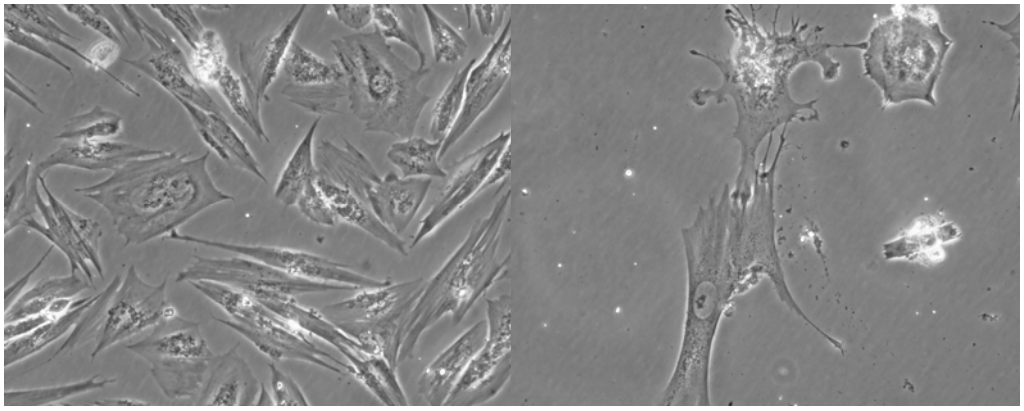

Fig. 26: Melanoma M17. Day 66. Spindloid, dendritic cells.

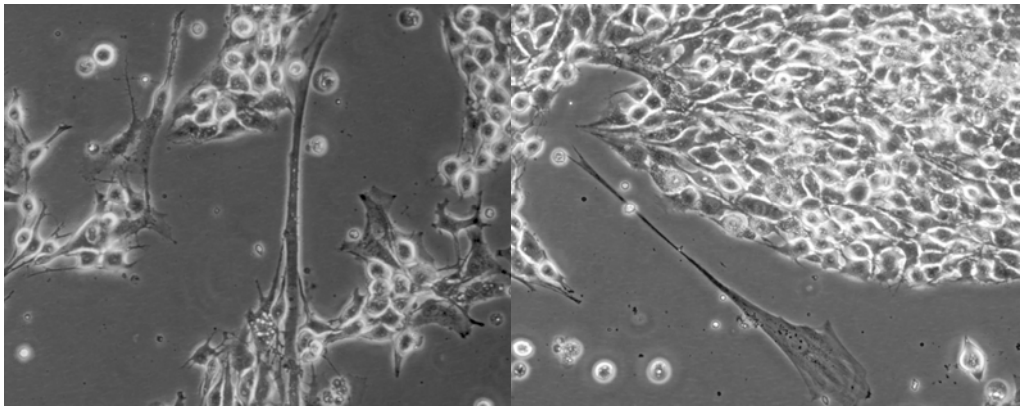

Fig. 27: Control melanoma cell line. Amelanotic, small, polygonal cells growing in sheets. Occasional dendrites.

First adherence of the suspended cells was observed between 2-18 days after isolation (Table 3). Cells of M9 adhered after 2 days already. By contrast, the cells of M12, which died for unknown reasons after 2 divisions, took 40 days until they showed evidence of adherence. Once single cells were adhered to the culture dish, their division lead to an accumulation of adhered cells in close relationship. In general, multiple islands of cell clusters were present on the Petri dish after a while. The point of time when confluency of the cells on the culture dish was reached varied widely. The fastest growing cell culture was confluent after 10 days, whereas the slowest growing culture took 100 days after initiation. Even though the cell cultures grew with different characteristics, the speed of growth was relatively constant among one cell culture. M8 was the slowest culture. It was the last one to reach confluency and continued to grow very slowly after being sub cultured.

Table 3: Starting days of adherence and confluency after initiation of the cell cultures.

Melanoma	Day of adherence	Day of confluency
M1	6	10
M4	8	42
M8	7	100
M9	2	36
M12*	40	died before
M14	10	60
M16	18	41
M17	13	56

*) Cell culture that died after 2 divisions

5. Discussion

With the described method for isolation and cultivation it was possible to successfully isolate and cultivate 7 grey horse melanomas. Cellular growth properties and cell morphology were observed *in vitro* and described for the first time. The donor horses were selected according to their availability and owners consent only. No consideration was given to breed, age, gender, localization, or size of tumor. Six different breeds contributed to the 7 successful cell lines. The distribution of gender (4 geldings, 2 mares, and 1 stallion) reflects the general horse population seen at the Equine Hospital at the University of Zurich. The age of the animals ranged from 6-20 years, representing the known age span of first melanoma appearance in grey horses. No obvious impact of breed, age, and gender of the donor horses on the outcome of a successful cell culture was noted.

The surgical protocol for standing excision served the purpose. Sedation and analgesia of the horses with detomidine and butorphanol was sufficient to allow excision of the tumors in a quiet and efficient matter. No adverse side effects were observed in any of the horses. Apart from occasional dehiscence of solitary sutures, wound healing was uncomplicated in all horses. No further medical or surgical treatment was necessary. It is of paramount importance that the surgical excision is carried out under aseptic conditions. Not only for an uncomplicated wound healing in the patient but primarily to ensure sterility of the tumor tissue. In primary cell cultures any source of contamination must be avoided at all means. Potential infectious agents could be carried into the cell culture laboratory with the primary tissue. Infected cell cultures must be discarded immediately. Normally they can not be saved and they put other cultures in the same incubator in danger.

The tumor material was immediately placed in sterile D-PBS with 1% Antibiotic Mixture at room temperature and transported to the cell culture laboratory within 15 to 20 minutes. The effects of transporting the tissue in another type of sterile fluid, the addition of various nutrients, antibiotics, antimycotics, and keeping the tissue at body temperature at all times remains unknown and requires further studies. There might be an improvement in the outcome if the tissue was minced into little pieces already at the time of excision. This results in a larger surface area for equilibration with the D-PBS. As we were successful in seven tumors we believe that our procedure is sufficient and does not necessarily need adaptations. The faster a tissue is processed after collection the more viable its cells are.[72] Even though 15 minutes is a

short time between excision and isolation we believe direct processing of the melanoma would yield an even better outcome. On the contrary, it must be assumed that prolonged storage time of several hours results in more non-viable cells and therefore a worse outcome. The effect of freezing the tumor tissue in liquid nitrogen and subsequent isolation after several days, weeks, or even months must be studied further. Immediate freezing would allow the collection of melanomas independent of time or place, for example at night time or in an abattoir. Collecting tumors from slaughtered horses would be an advantage. It would facilitate rapid and sterile tumor collection yielding a large amount of viable tissue. The fact that the excisions had to take place in close proximity to the cell culture laboratory and that all involved staff and necessary equipment had to be ready at the same time made it difficult to collect a large number of tumor samples within a reasonable time frame.

The procedure would be facilitated further if smaller amounts of tumor material would be enough for processing, allowing fine needle aspiration biopsies or excisional biopsies to be adequate instead of excising whole tumors. Horse owners are less hesitant to have a small biopsy taken from their horse than excising an entire tumor. For the horse a biopsy is less unpleasant than surgical excision. The fact that the tumor remains for further analyses before, during, and after prospective therapy, represents an additional advantage.[66]

Both applied enzymatic digestion protocols yielded the same results. If incubation was successful in a tumor, both attempts worked. However, if time allows, the protocol could be prolonged for both enzymes or the minced tissue could be incubated overnight. The effect of these changes to the procedure might improve isolation and should be studied further. Using dispase and collagenase as digestion enzymes worked well. While other enzymes might be used instead, we did not examine the effects of different enzymatic assays. Collagenase is costly, therefore it would be desirable to replace it with a less expensive enzyme. RPMI 1640 growth medium must be present at all times to ensure adequate nutrition and equilibration of the tissue and isolated cells. The temperature of the water bath should be at body temperature for optimal enzymatic conditions. Colder temperature demands longer incubation times resulting in a higher risk of cell death. Higher temperature kills the cells and might slow down or even stop enzymatic disaggregation. To filter the cell suspension before the last centrifugation proved useful. Large clumps of undigested tissue and remaining connective tissue could be discarded resulting in a relatively pure cell suspension. Maintenance of the cell

cultures was conducted according to standard cell culture practice for mammalian cells. Because many factors contribute to successful growth of a culture it is impossible to assess the influence of any single factor.

Temperature, humidity, growth medium, supplements, oxygen and carbon dioxide concentration, pH, osmolality, culture flask or dish, and other factors may have influenced the outcome of our melanoma cell cultures. These effects need further investigation to decide if better growth conditions for equine melanoma cells are possible. Seven of 17 tumors yielded continuously growing cell lines. The success rate of establishing melanoma cell lines of 41%, or 54% not including the contaminated cultures, contrasts reported success rates of 70-90% in human medicine,[66] or 81% in swine.[44] Procedures in human medicine are very advanced because of longstanding experience and a large availability of tumor material. Obstacles in our study were the difficulty of recruiting a large number of donor horses due to owner's hesitance and the strict requirements for the procedure we set. No information about experience with grey horse cell culture was found in the current literature. For these reasons we are satisfied with our success rate for this point of time. However, further studies are needed to optimize collection techniques of tumor material and improve cell culture methods. Melanoma M12 cells died after 2 divisions for unknown reasons. The cells of this melanoma had an atypical appearance and growing pattern when compared to the successful seven. Either they experienced programmed cell death or they needed different growing conditions. The exact cause of cell death is only speculative. The 4 cell cultures that were contaminated illustrate the importance of avoiding infection. They could not be salvaged and had to be discarded before any adherence or growth was observed. It is unknown if without contamination those cultures would have been successful. The time of adherence varied between 2-18 days in the successful cultures and the discarded cultures did not survive for more than one week. Because all cultures showed signs of contamination on the same day it was assumed that the infectious agent had been transmitted from one contaminated culture to the others by means of contaminated media, instruments or non-sterile handling.

Six tumors failed to grow *in vitro*. The inability to establish cell lines from those tumors may have been to the result of medium constituents, selection for non-viable cells in the tumors, or any cell characteristics requiring handling that was not adequately met. Cells grow *in vivo* with close interactions with adjunct cells. *In vitro* those mechanisms are lacking and therefore

growth conditions are not ideal. In swine it was shown that samples, which failed to grow, were derived from ulcerated, melanin-exuding lesions which consisted of free melanosomes and few cells.[44] We experienced similar effects (see below).

The tumor site did not seem to influence the outcome of a cell culture. Even though tumors from the perianal region and tail predominated in the horses that were presented, one of the successful tumors originated from the head. Intradermal tumor location did not affect outcome, however, both surgical excision and further processing in the cell culture laboratory were more complicated. It was faster and easier to get rid of connective and fat tissue but dissection of the frequently strongly attached epidermis from the melanoma was more difficult. Different consistencies of the tumors lead to several problems. Very firm tumors were hard to mince into little pieces and therefore took longer to process until being incubated with growth medium in the first dissociation step. Several of the unsuccessful tumors were soft and necrotic in the center, with black fluid leaking out. Only an intact outer layer was present (Fig.13). Therefore, it was of benefit to discard all necrotic tissue and use peripheral, "healthy" tissue only. However, this is difficult to assess visually and requests experience by the handler. Free necrotic tissue and pigment could potentially disturb cell growth in the final cell culture. Pigment could not be discarded by filtering. It tended to adhere to the culture dish and was hard to remove by changing the culture medium. Hence, it was important to avoid adding free pigment to the cell culture in the first place. In the authors' experience the smaller a tumor, the better were its processing characteristics. It appeared that the larger tumors contained more necrotic areas and had worse growing properties, such as observed in melanoma M2. Therefore it is recommended to excise whenever possible small, well confirmed, subcutaneous melanomas.

All submitted melanomas were confirmed histologically. In the tumors of the unsuccessful group there were tendencies toward more central necrosis and/or a larger amount of connective tissue. The histologic findings corresponded to the macroscopic findings discussed above. Abundant connective tissue interfered with the processing of the melanoma and yields a proportionally smaller amount of isolated melanoma cells. Fibroblasts and stromal tissue cells are difficult to filter away from the tumor cells, which may result in fibroblast overgrowth.[66] The cells of the unsuccessful tumors were not dendritic in shape, whereas some of the successful cells had dendrites. Dendrites are an important organ of melanocytes *in vivo*. It

enables the cell to interact with neighboring cells and transfer melanin.[73] There might be a better potential for *in vitro* growth for cells that have dendrites *in vivo* already.

Cells in the unsuccessful group were generally larger than in the successful group. Size could have an impact on a cell's ability to survive when isolated and grown in a cell culture. However, the reasons for different behavior must be studied further. Improved histologic characterization would have been possible if strongly pigmented melanomas would have been bleached. This procedure enables better interpretation of the nuclei in regard to morphology, pleomorphism, and presence of mitotic figures resulting in a more reliable decision on malignancy.[12, 74] Cellular morphology was similar in most melanoma cells *in vitro*. Even though cancer cells are degenerated, the spindle shape and dendritic processes in the primary cultures may reflect characteristics of what is known of normal melanocytes *in vivo*. Melanocytes are located in the basal cell layer of the epidermis and in hair follicles.[73] They have dendritic processes that intussuscept into the surrounding keratinocytes. Horses have one melanocyte per 10 to 20 keratinocytes.[75] Synthesis of melanin takes place within specialized intracellular organelles called melanosomes. After production the pigment is stored in granula and transported to the dendritic processes. There, the melanin granula are transferred to adjacent cells.[76] In keratinocytes melanin granules are often clustered dorsal to the nucleus.[75] The cultured melanoma cells showed a similar tendency. Dark brown pigment was produced and excreted extracellularly. The pigment clustered in the center of a group of cells. The presence of dark brown pigment and the cell's typical behavior strongly suggests that the cells really were melanoma cells. Up-regulation of melanogenesis in melanoma is unknown in horses, but has been shown in other species.[77]

All grey horse melanomas were dark black on cut surface and there was obvious pigment production in the cell cultures. This observation would support this theory in horses as well. Beyond primary culture the cells seemed to be smaller and less dendritic. They appeared less melanotic or even amelanotic. It appeared that the melanin-producing ability was lost after the first passages. Normal melanocytes are in close relationship with keratinocytes, called the epidermal melanin unit.[73, 75] However, melanoma cells grow in an expansive and uncontrolled manner. Whether melanoma cells *in vitro* would profit from interaction with some kind of other cell type is unknown. In our cell cultures growth was possible without any scaffold or support. The addition of other cells could interfere with characterization experiments and therefore is

not recommended. Initially, there was a wide range of adherence and speed of cell growth. In porcine melanoma, growth characteristics of cell cultures varied with the age of the swine from which the tumors were obtained.[44] Cell cultures obtained from swine younger than 2 months grew steadily and rapidly with cell division ceasing after 6-8 passages. Cell cultures of tumors obtained from swine aged 3 months or older showed a biphasic growth pattern with an early slow growth rate. However, they lived longer and terminated growth after 18-20 passages. The effect of the donor horse's age on cell culture growth characteristics remains to be examined.

The objectives of our study were to establish a novel method for isolation of grey horse melanoma cells and to study the histology of the tumor and the morphology of the cell line originating from it. Limitations in our study were that several routine examinations of cell lines were not performed. This includes population doubling times, number of passages until termination of cell division and growth, and electron microscopy examinations. In melanoma cells melanin-synthesizing characteristics such as DOPA-oxidase histochemistry and melanin spectroscopy of the culture medium would have been of interest.[44]

6. Conclusion

A method to successfully isolate grey horse melanoma cells and to establish cell lines is described. Seven permanently growing cell lines were established, representing a success rate of 41%. Tumors of various locations in horses of different breeds, ages, and genders were cultivated with no apparent difference in success. The cells adhered after 2-18 days and reached confluency after 10-100 days. *In vitro* the cells appeared dendritic and produced pigment, which clustered extracellularly. Further studies to improve the described protocol should include examination of different excision protocols, changes in tumor storage, and the exploration of various growing conditions on the outcome of cell cultures. The cell lines that were established should be characterized with experiments using specific melanoma antigens. If this method of obtaining and establishing tumor cell lines of grey horse melanomas reaches a near 100% success rate, investigations on immunobiologic and pathobiologic features of equine melanoma are possible and research on effective melanoma therapy in horses can be advanced.

7. Footnotes

a. Roswell Park Memorial Institute 1640 medium (1x) liq., 21870-076, Invitrogen Corporation, Paisley, UK
b. L-Glutamine 200mM, liq., 25030-024, Invitrogen Corporation, Paisley, UK
c. Sodium pyruvate 100mM, liq., 11360, Invitrogen Corporation, Paisley, UK
d. Penicillin/Streptomycin Sol. (5000:5000), liq., 15070-063, Invitrogen Corporation, Paisley, UK
e. ES cell qualified fetal bovine serum, 16141-079, Invitrogen Corporation, Paisley, UK
f. Phosphate-buffered saline, (1x), liq., 10010-015, Invitrogen Corporation, Paisley, UK
g. Dispase, 17105-041, Invitrogen Corporation, Paisley, UK
h. Sodium chloride, 71378, FLUKA, Sigma-Aldrich Corporation, Buchs
i. Tris (Hydroxymethyl) aminomethane, 44333, BIOSOLVE, Valkenswaard, Netherlands
j. Potassium chloride, P9333, Sigma-Aldrich Corporation, Buchs
k. Hydrochloric acid, 320331, Sigma-Aldrich Corporation, Buchs
l. Sodium phosphate dibasic, S7907, Sigma-Aldrich Corporation, Buchs
m. Potassium phosphate monobasic, P0662, Sigma-Aldrich Corporation, Buchs
n. Calcium chloride, C1016, Sigma-Aldrich Corporation, Buchs
o. Tris-buffered saline, own stock
p. Collagenase from Clostridium histolyticum, C2674, Sigma-Aldrich Corporation, Buchs
q. Ethylenediaminetetraacetic acid disodium sulfate (EDTA), 03679, FLUKA, Sigma-Aldrich Corporation, Buchs
r. Steritop-GP filter unit, SCGPT05RE, Millipore, Billerica, MA, USA
s. Puradisc™ FP30/0.45/0.2 CA-S, Whatman Switzerland GmbH, Bottmingen
t. Domosedan, Pfizer AG, Zürich
u. Morphasol, Dr. E. Graeub AG, Bern
v. Fluniximin, Berna Veterinärprodukte AG, Bern
w. Lidokain Hyaluronidase 2%, G.Streuli & Co. AG, Uznach
x. Formalinlösung 4% gepuffert pH7, Kantonsapotheke Zürich
y. D-PBS (1x), liq., without Ca, Mg, Phenol Red, 14190-094, Invitrogen Corporation, Paisley, UK
z. Tissue culture dish 100/20mm, 664160, Greiner Bio-One GmbH, Frickenhausen, Germany

aa. BD Falcon™ Conical Centrifuge Tubes, 15ml (352096) and 50ml (352070), BD Biosciences, Allschwil

bb. BD Falcon™ Cell Strainers, 70um, 352350, BD Biosciences, Allschwil

cc. Trypsin-EDTA solution, T3924, Sigma-Aldrich Corporation, Buchs

dd. Cell culture freezing medium, 11101-011, Invitrogen Corporation, Paisley, UK

ee. Nunc CryoTubes™, 366656, Nunc GmbH & Co. KG, Wiesbaden, Germany

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10. Curriculum Vitae

Name	Nadine Metzger
Date of birth	23rd of January, 1976
Place of birth	Fribourg
Nationality	Swiss
Native place	Kirchberg SG

1983 – 1988	Elementary school Uhwiesen, ZH and Ettingen, BL
1988 – 1995	High school Therwil, BL and Oberwil, BL
1995	Graduation (Maturität Typus C) Oberwil, BL
1996 – 2001	Study of veterinary medicine, University of Zürich, Zürich
1998 – 1999	Study of veterinary medicine, Universidad de Córdoba, Córdoba, Spain
2001	Graduation (Staatsexamen) University of Zürich, Zürich
2001 – 2002	Internship in equine neonatology and critical care, The Ohio State University, Columbus, OH, USA
2002 – 2003	Internship in equine surgery and internal medicine, Marion duPont Scott Equine Medical Center, Virginia Polytechnic Institute and State University, Leesburg, VA, USA
2003	Research assistant, Bogar AG, Zürich
2003 – 2004	Assistant veterinarian in small animal clinic, Dres. E. Meng and D. Damur, Chur
2004 – 2006	Assistant veterinarian in equine internal medicine, Equine Clinic, Vetsuisse Faculty University of Zürich, Zürich